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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

We found that DIP1, a novel 45 Kda nuclear protein recently identified in our laboratory, is frequently expressed in primary human breast cancers and breast cancer cell lines. However the level of expression o DIP1 in breast cancer cells appears to be lower than that in normal mammary epithelial cells. Overexpression of DIP1 in MCF-7 human breast cancer cells inhibited colony formation and cell proliferation but did not inhibit specific phases of the cell cycle. In mechanistic studies we found that DIP1 inhibits the transcriptional activities of the cyclin D1, c-fos, SRE, and NF Kappa B promoters. Studies with mutant and truncated forms of DIP1 indicated that the HLH domain present in this protein plays an important, but not exclusive, role in these inhibitory effects. DIP1 also markedly inhibited the stimulation of cyclin D1 promoter activity obtained with TSA, an inhibitor of histone deacetylase (HDAC), and DIP1 co-immunoprecipitated with HDAC1 and HDAC3. Therefore, DIP1 is a putative tumor suppressor gene in breast cancer cells that appears to inhibit gene transcription by affecting the process of histone acetylation. It may provide a molecular target for breast cancer therapy.

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INTRODUCTION

Breast Cancer is the most common form of cancer in woman in the United States. Each year about 180,000 new cases of breast cancer are diagnosed, and 44,000 women die from this disease. At a world-wide level breast cancers afflict over 900,000 women per year. It appears that the majority of breast cancers result from a combination of reproductive, environmental (including dietary), lifestyle, and genetic susceptibility factors, but in many cases the precise factors remain to be identified. Also, like other types of cancer, the development of breast cancer is a multistage process that involves the progressive acquisition of mutations and/or aberrant expression of several genes. The genes involved can be highly diverse with respect to their cellular functions. When cells divide and multiply they go through a clock-like mechanism called the cell cycle. One of the genes that plays a critical role in controlling the cell cycle is called *cyclin D1*. In previous studies our laboratory and other investigators found that there is frequently an abnormal increase in the expression of this gene in human breast cancers. In recent studies we discovered a new gene called *dip1* (Ref 1). This gene encodes a novel 45kDa nuclear protein that contains a HLH domain characteristic of certain transcription factors (Fig. 1). The overall purpose of this project is to determine whether the *dip1* gene plays a critical role in the development of breast cancer. The results obtained could suggest novel strategies for breast cancer prevention and therapy.

BODY

Hypothesis/Purpose

The overall hypothesis tested in this proposal is that DIP1, a novel HLH protein recently identified in our laboratory, plays a critical role in the development of human breast cancer. If this hypothesis proves to be correct, then DIP1 or proteins that interact with DIP could provide novel targets for breast cancer chemoprevention and/or therapy. The level of expression of DIP1 in breast cancers might also provide a biomarker for prognosis.

Research accomplished on specific tasks, during this project.

1. Task 1. Determine whether the DIP1 gene is amplified, rearranged and/or altered in its level of expression in human breast cancer cells.

Proteins were extracted from several human breast cancer cell lines and from 20 frozen tissue samples of primary human breast cancers. Using an antibody to DIP1 generated in our laboratory we then examined the expression of the DIP1 protein by the technique of Western blot analysis. We found that all of the breast cancer samples expressed fairly similar levels of the DIP1 protein. It is of interest that the normal human mammary epithelial cell line MCF-10F expressed higher levels of DIP1 than the human breast cancer cell line MCF-7. These findings are consistent with our hypothesis that this protein may play an important role in breast cancer, perhaps as a tumor suppressor gene.

Southern blot analyses of the DNA extracted from these same breast cancer samples did not reveal any evidence of amplification or gross rearrangements of the *dip1* gene (data not shown).

2. Task 2. Determine the biologic effects of DIP1 by developing derivatives of MCF-7 cells with altered levels of expression of this protein.

The DIP1 cDNA was inserted in the "sense" or anti-sense" orientation into the expression vector pCEP4. We also used a DIP1 cDNA containing a point mutation at residue 28 (Leucine⇒ Alanine). This mutant is designated "Dipmut." This region of the DIP1 protein is of interest because it contains a LXXL motif (Fig. 1) which is present in several nuclear transcription co-activators (8). These vectors were then used to transfect MCF-7 cells and clones were isolated from the cells transfected with the "sense" DIP1 or DIP1mut construct, that stably expressed increased levels of the DIP1 protein, when examined by western blot analysis. We were not, however, able to obtain derivatives of the MCF-7 cells transfected with the antisense constructs that expressed decreased levels of the DIP1 protein.

Cell proliferation studies (MTT) assays indicated that the increased expression of DIP inhibited the growth of MCF-7 cells. Inhibition was also observed with the DIP mut construct

although this was somewhat less than that obtained with the wild type protein. Representative studies, employing as a control a derivative that was transfected with only the empty pCEP4 vector, are shown in Figure 2B.

To exclude the possibility that these results reflected spontaneous clonal variation or secondary changes during selection of the derivatives, we also did transient transfection assays to determine the effects of the DIP1 and DIP1mut constructs on the colony forming ability of MCF-7 cells. The stained plates are shown in Figure 2A. Both the DIP1 and DIP1mut constructs markedly inhibited colony formation and again, the effect was greatest with DIP1. Colony counts indicated that DIP1 caused about 94% inhibition and DIP1mut about 84% inhibition of colony formation.

Cell cycle analysis did not reveal any major differences between the DIP1 overexpressing MCF-7 cell and the vector control cells with respect to the percent of cells in the G1, S or G2/M phases of the cell cycle (Figure 3). Therefore, it appears that the inhibition of growth by Dip1 is not exerted on a specific phase of the cell cycle.

These studies establish DIP1 as a protein that inhibits rather than stimulates the growth of breast cancer cells, which is consistent with the above mentioned finding that the level of expression of DIP1 in MCF-7 cells is lower than in the normal human mammary epithelial cell line MCF-10F. These findings, do not, however, indicate the mechanism of action of this protein, which is the goal of the studies described below.

Task 3. Studies on the mechanism of action of DIP1, emphasizing its role in gene transcription.

We originally identified DIP1 by its ability to bind to cyclin D1 in the yeast two-hybrid system (1). However, we have not been able to consistently demonstrate, by co-immuno-precipitation, that it binds to cyclin D1 in breast cancer cells. Therefore, we have pursued other directions to elucidate its mechanism of action, focusing on possible effects on gene transcription since DIP1 contains a HLH domain frequently found in transcription factors (2-7).

To assess possible effects on gene transcription we did transient transfection-reporter assays, with various promoter elements linked to a luciferase reporter. Transfection assays were done in the absence or presence of the pCEP4 vector itself, the DIP or the DIP1mut vectors. The results indicated that both DIP1 and DIP1mut markedly inhibited both the cyclin D1- and c-fos-luciferase reporters (Figure 4). On the other hand, only slight inhibition by DIP1 or DIP1 mut was seen with a CMV-beta gal reporter (data not shown). The latter result suggests that DIP1 inhibits the transcription of specific genes and is not simply a non-specific inhibitor of transcription. DIP1 also inhibited the transcription of an estrogen response element (ERE)-luciferase reporter, but this effect was quite variable (data now shown).

As indicated in Figure 1, Dip1 contains several motifs which, beginning at the amino

terminus include: the LXXL and leucine zipper motifs, a HLH domain, and an acidic region. The LXXLL motif at the amino terminus is present in several nuclear transcription co-activators (8). As described above, we found that a mutant of Dip1 with a point mutation at residue 28 in which the first leucine of the LXXL motif was replaced with alanine still inhibited the growth of MCF-7 cells (Figure 2) and also inhibited cyclin D1 promoter - and c-fos promoter-activity (Figure 3), although these effects were slightly less than those seen with wild type Dip1. To further explore the functions of each of these motifs, with respect to effects on transcription, we generated a series of truncated mutants of Dip1 (Figure 5A), which were linked to the expression vector pCEP4.

These constructs were then assayed for their relative abilities to inhibit cyclin D1 promoter-luciferase activities in transient transfection assays in MCF-7 cells. Since β-catenin is known to stimulate the activity of the cyclin D1 promoter (9) it was used as a positive control (Figure 5B). Initially we tested an amino terminal fragment which included the HLH domain (NT-HLH) and a carboxyl-terminal fragment that lacked the HLH domain (CT) (Figure 5B). When compared with the intact Dip1, NT-HLH still had strong inhibitory activity, but this was somewhat less than that of Dip1, and the CT fragment gave partial inhibition. When, however, we tested a CT fragment that included the HLH domain (HLH-CT) the inhibition was almost as great as that of NT-HLH fragment. These findings suggest that the HLH domain plays an important role in the ability of Dip1 to inhibit transcription in this system, although there also appears to be independent inhibitory activity in the CT fragment that lacks the HLH domain.

We then tested a series of 7 short fragments of DIP1 each of which contained individual motifs or intervening regions, which were designated T1 through T7 (Figure 5A). None of these displayed significant inhibitory activity of cyclin D1 promoter-luciferase activity(Figure 5C), including the fragment encoding the HLH domain (T4). Presumably, these results reflect the need for multiple regions of the Dip1 protein for an inhibitory effect on transcription and/or the need for an appropriate secondary structure of the protein.

We next examined the specific regions or elements of the cyclin D1 promoter that are required for Dip1 to exert its inhibitory effects on transcription, by using a series of 5' to 3' deletions of the cyclin D1 promoter in promoter-luciferase constructs, testing each construct in transient assays in the presence of wildtype Dip1 or vector control DNA. We found that Dip1 strongly repressed both -1745CDLUC and -963CDLUC promoter activity, both of which contain the E box sequence, which is present at -546 in the cyclin D1 promoter (10). However, Dip1 also strongly repressed -261CDLUC and -163CDLUC promoter activities, even though these constructs do not contain the E-box sequence. Therefore, even though Dip1 resembles in some respects ID proteins (1), which can inhibit E box promoter activity (11), the inhibitory effect of Dip1 on transcription is not confined to the E box element. Indeed, we found that even the activity of the extensively truncated cyclin D1 promoter -22CDILUC was inhibited by Dip1, as were constructs in which there were point mutations in either the Sp1, CRE or NF-Kappa B elements (Figure 6).

In view of the latter results, it was of interest to also examine the effects of some of the mutant and truncated forms of Dip1 described in Figure 5A on the activity of the -22CDLUC reporter. As with the -1745 CDLUC reporter (Figure 5B), we found that the Dipmut and NT-HLT constructs had the greatest inhibitory activity, but the CT construct also had some inhibitory activity (Figure 7). We specifically examined the effects of Dip1 on a NF-Kappa B-luciferase reporter in transient transfection assays in MCF-7 cells, and found that it also markedly inhibited this reporter (Figure 7B). However, for reasons that are not apparent the Dip1mut, NT-HLH, CT and HLH-CT derivatives of Dip1 were not as inhibitory with this reporter (Figure 7B) as they were with the -22CDLUC (Figure 7A) and the -1745 CDLUC (Figure 6B) reporters. On the other hand when we used a SRE-LUC reporter in MCF-7 cells Dipmut, NT-HLH and HLH-CT had relatively strong inhibitory activity when compared to wild type DIP1 (Figure 7C). Taken together, these results suggest that the HLH domain of DIP1 plays an important, but probably not exclusive, role in inhibiting transcription from several promoter elements.

There is considerable interest in the role of histone acetylation in stimulating the transcription of specific genes (12-14). The extent of histone acetylation reflects a dynamic balance between the extent of histone acetylation, (for example, on residues K9, and K14 of histone H3) by a series of histone acetylation (HATs) enzymes versus the extent of histone deacetylation by a set of histone deacetylases (HDACS) (for review see 15,16). Therefore, we repeated the above studies with the cyclin D1 promoter- and c-fos promoter-luciferase reporters, in the absence and presence of trichostatin A (TSA), an inhibitor of HDAC (17,18). Figure 8A indicates that when added at 50-200 nM TSA caused a marked dose dependent stimulation of both *c-fos* and cyclin D promoter transcription. In both cases this stimulation was markedly inhibited by DIP1.

The above results suggested that DIP1 might interact with a component of transcriptional complexes that affects the state of histone acetylation. Therefore, we examined the possibility that DIP1 binds to one or more HDACs, thereby possibly enhancing their activity or enhancing their recruitment to chromatin complexes. MCF-7 cells were transiently transfected with DIP1, flag-tagged HDAC1 or flag-tagged HDAC3 plasmids. Total protein extracts were prepared 24 hours later and immunoprecipitated with an anti-flag antibody (to pull down HDAC3). The immunoprecipitates were then examined by western blot analysis using a DIP1 antibody. In a reciprocal study cos7 cells were transiently transfected with HA-tagged DIP1, HA-tagged Dipmut or flag-tagged HDAC1. Total protein extracts were prepared 24 hours later and immunoprecipated with a HA antibody (to pull down DIP1) and the immunoprecipitates then examined by western blot analysis with the anti-flag antibody, to detect HDAC1. The results of both studies are shown in Figure 8B. They indicate that DIP1 binds to both HDAC1 and HDAC3, and that Dipmut also binds to HDAC1.

In view of the above results it was of interest to examine the possibility that histone acetyltransferases (HAT) might reverse the inhibitory effects of Dip1 on cyclin D1 promoter-luciferase transcriptional activity. However, in cotransfection assays we found that neither HAT p300 or HAT PCAF reversed the inhibitory effects of Dip1, Dip1mut, Dip1-NT or Dip1-CT on cyclin D-luciferase activity in MCF-7 cells (Figure 9).

4. Discussion/Relevance

The results we have obtained during this project provide strong evidence that the novel protein DIP1 acts by inhibiting the transcription of specific genes and that this appears to involve, at least in part, effects on the process of histone acetylation. This inhibitory effect on transcription explains why we found that overexpression of DIP1 inhibits the growth of MCF-7 human breast cancer cells. It appears, therefore, that Dip1 may be a tumor suppressor gene. The exact clinical relevance of these studies remains to be determined. However, our findings suggest that human breast cancers that have reduced or undetectable levels of Dip1 may be more aggressive. If in future studies this is found to be the case then Dip1 could provide a useful biomarker of prognosis. In addition, therapeutic strategies that cause increased expression of Dip1 or enhance its activity might be useful in the treatment of breast cancer.

Future Studies

Our future studies with funding from other sources will have two goals:

1) To more precisely define the roles of Dip1 in the control of gene transcription

2) To determine the clinical relevance of Dip1 expression in patients with human breast cancer to determine whether it is a useful biomarker of prognosis and a potential molecular target for therapy.

6. Figures 1-9 and Legends (Attached)

KEY RESEARCH ACCOMPLISHMENTS

1. We found that DIP1, a novel 45 Kda nuclear protein recently identified in our laboratory, is frequently expressed in human breast cancer. However, the level of expression of this protein appears to be lower in breast cancer cells than in normal mammary epithelial cells.

2. Overexpression of this protein in MCF-7 human breast cancer cells markedly inhibits growth.

3. Mechanistic studies provide evidence that DIP1 inhibits the transcription of specific genes by affecting the process of histone acetylation. The HLH domain in DIP1 plays a critical role in this process but other regions in the DIP1 protein also appears to play important roles.

REPORTABLE OUTCOMES

Manuscripts/Abstracts

1. Yao Yao, Yuichiro Doki, Wei Jiang, Masaya Imoto, V.S.Venkatraj, Dorothy Warburton, Regina M. Santella, Binfeng Lu, Llunbiao Yan, Xiao-Hong Sum, Tao Su, Jingqing Luo and I. Bernard Weinstein. Cloning and characterization of DIP1, a novel protein that is related to the Id family of proteins. Exptl. Cell Research, **257**, 22-32, 2000.

2. Tao Su, Jingqing Luo, Yao Yao and I. Bernard Weinstein. Relevance of DIP1, a novel cyclin D1 binding protein to breast cancer. Annual Meeting of the American Association for Cancer Research, San Francisco, CA, April 2000.

3. Tao Su, Yao Yao, Richard Pestell and I. Bernard Weinstein. DIP1 is a novel inhibitor of transcription and a potential tumor suppressor gene in human breast cancer, manuscript in preparation.

Development of cell lines and other useful reagents.

1. Derivatives of MCF-7 that stably overexpress DIP1 or DIP1 mutant

2. Mutant and truncated forms of DIP1 that are useful for mechanistic studies

Training

During the course of these studies Dr. Tao Su, a post-doctoral research scientist, obtained extensive training in breast cancer research and in gene transcription.

CONCLUSIONS

A novel nuclear protein designated DIP1 has been identified that inhibits the growth and the transcription of specific genes in human breast cancer cells. These results suggest that DIP1 may be a tumor suppressor gene in human breast cancer, that could serve as a biomarker of prognosis and a molecular target for breast cancer chemoprevention and therapy.

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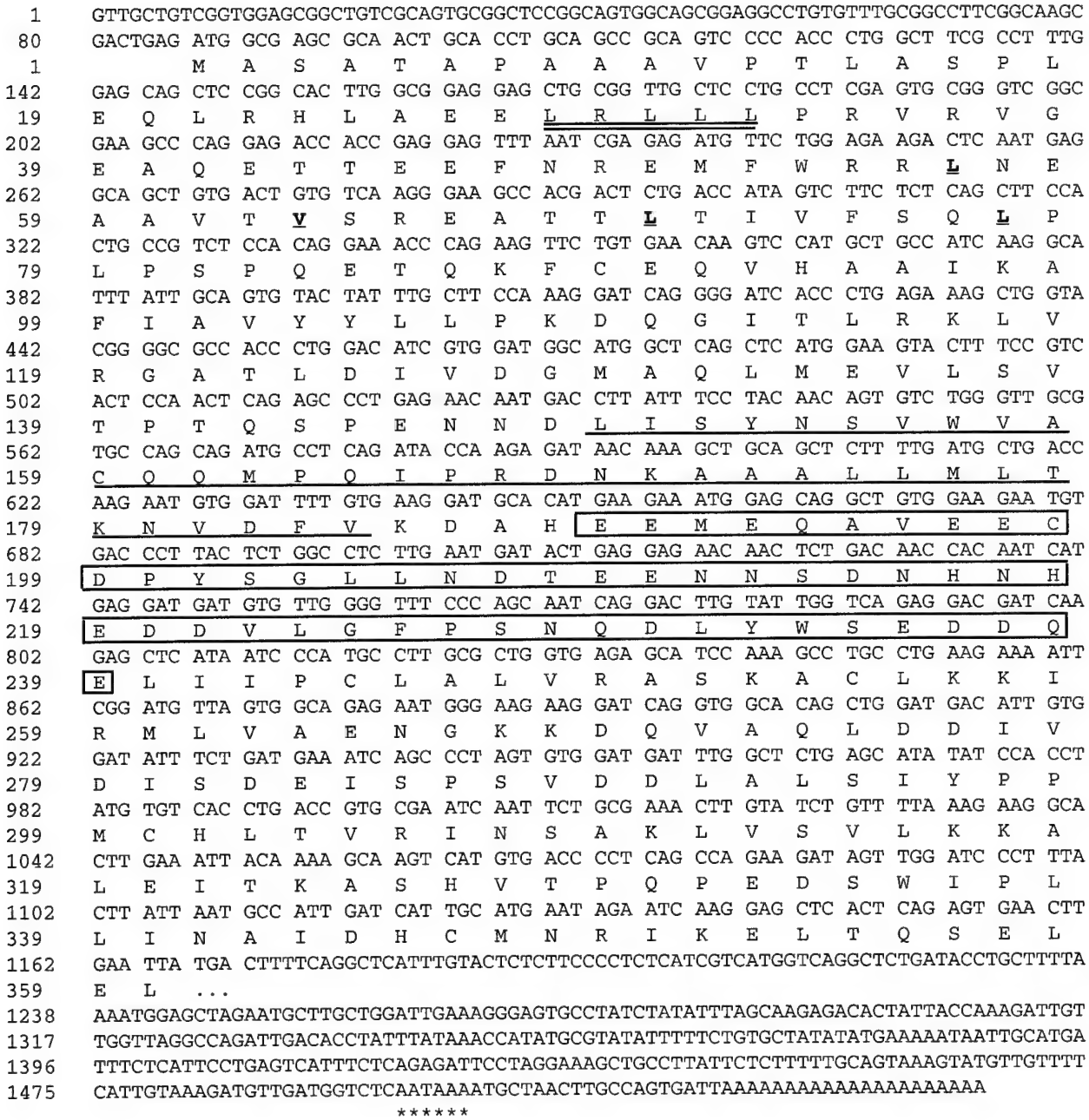
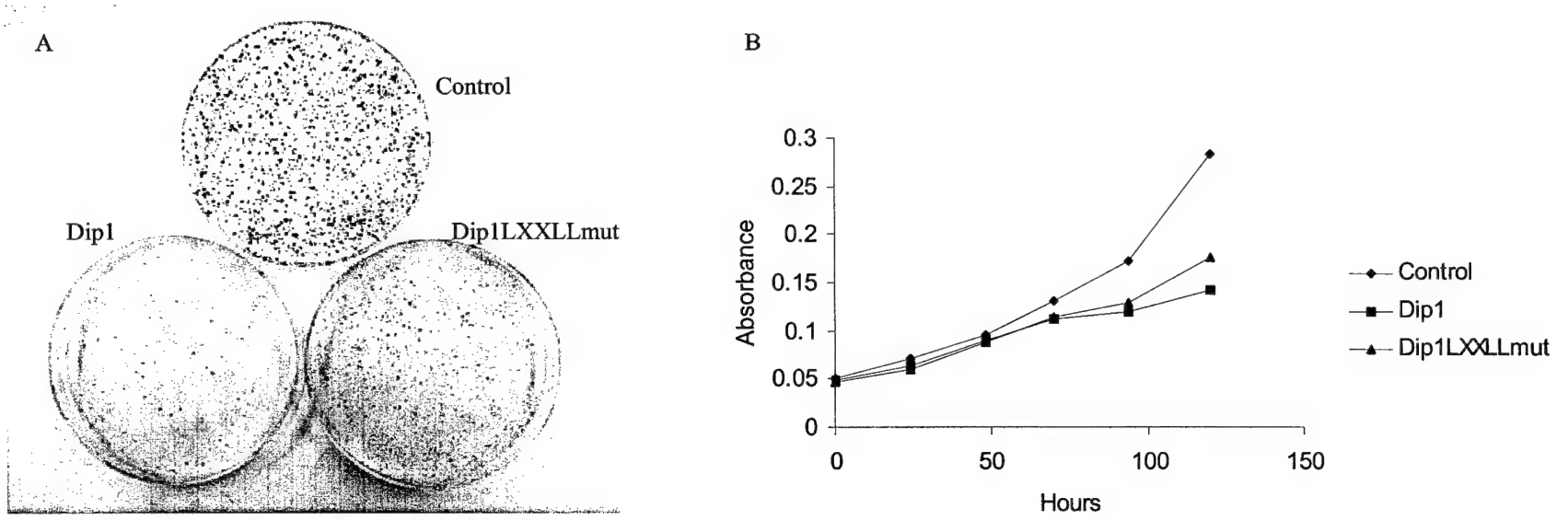


Figure 1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Control
Dip1
Dip1LXXLLmut
B
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0.25
0.2
0.15
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Absorbance
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50
100
150
Hours
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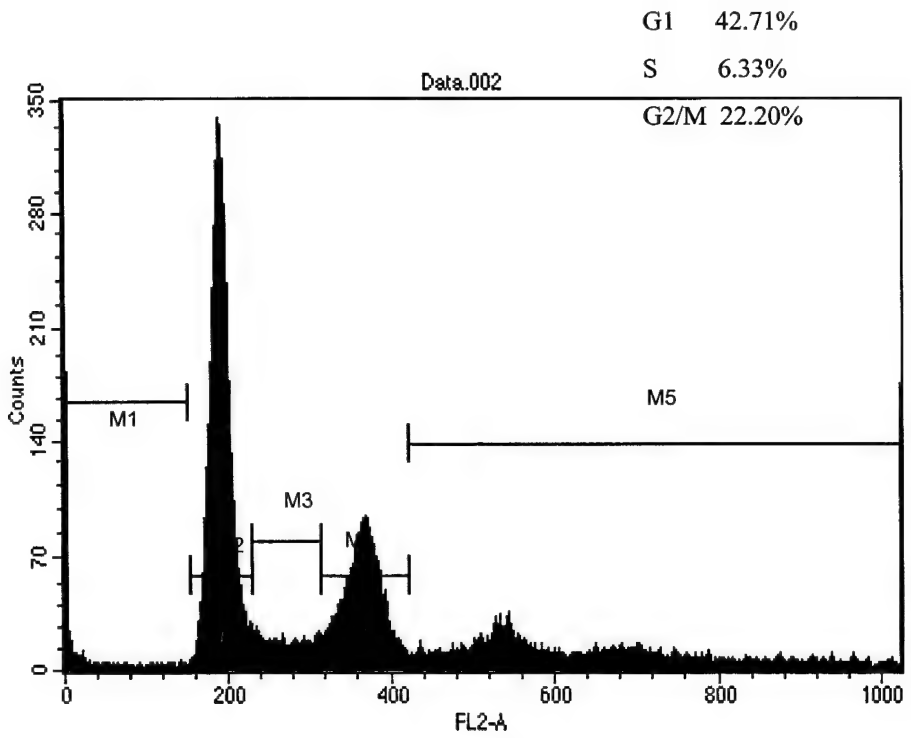
Figure 2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G2/M 22.20%
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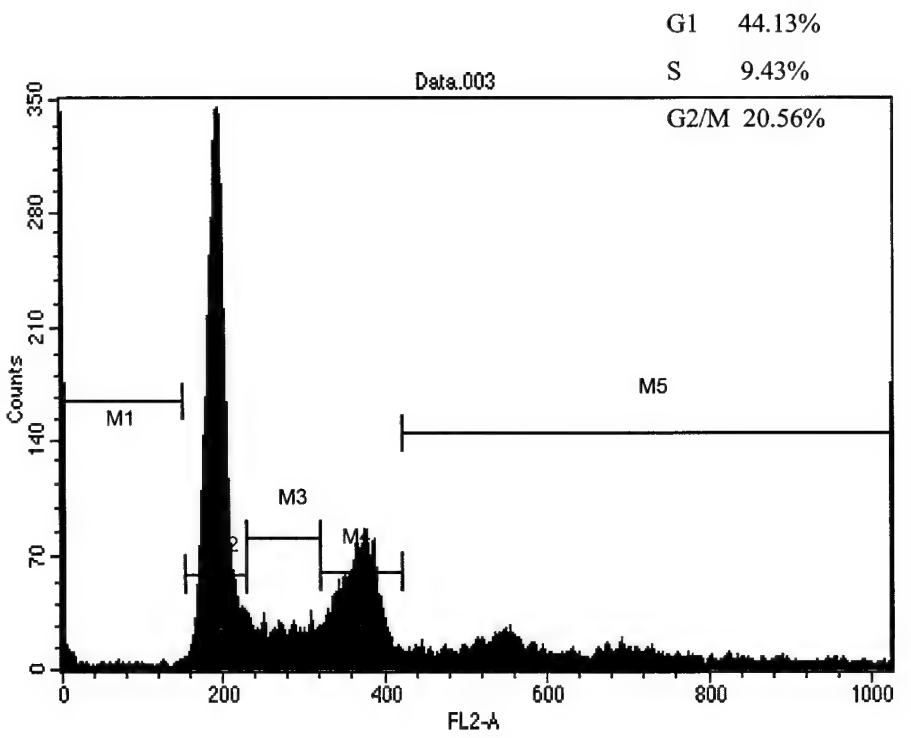
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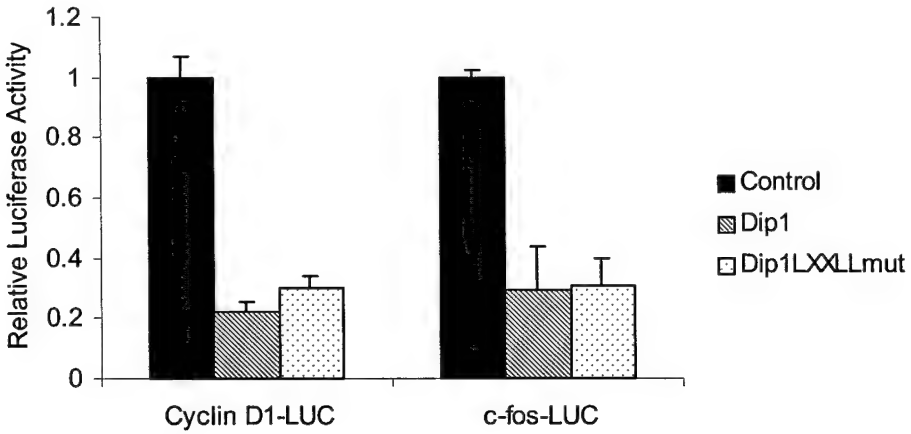
Figure 3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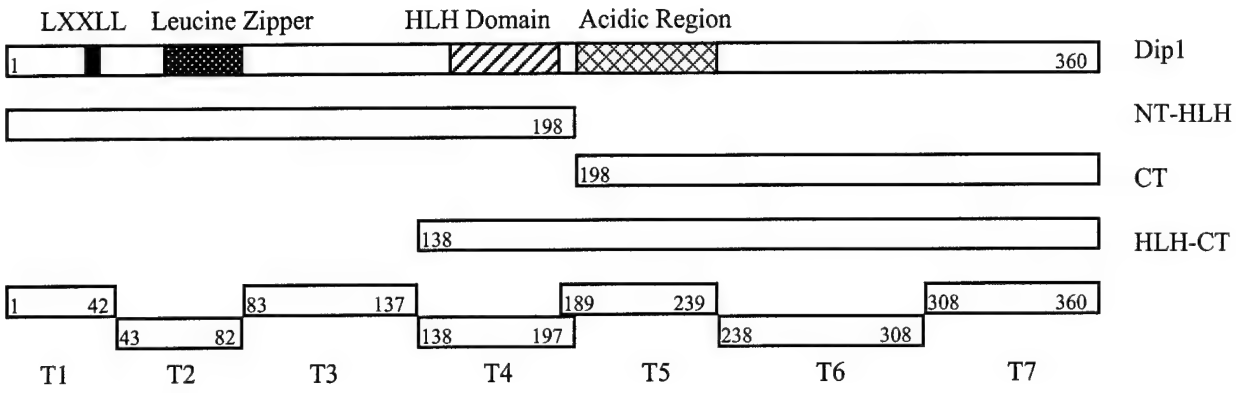
c-fos-LUC
Control
Dip1
Dip1LXXLLmut

Figure 4

A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238 308
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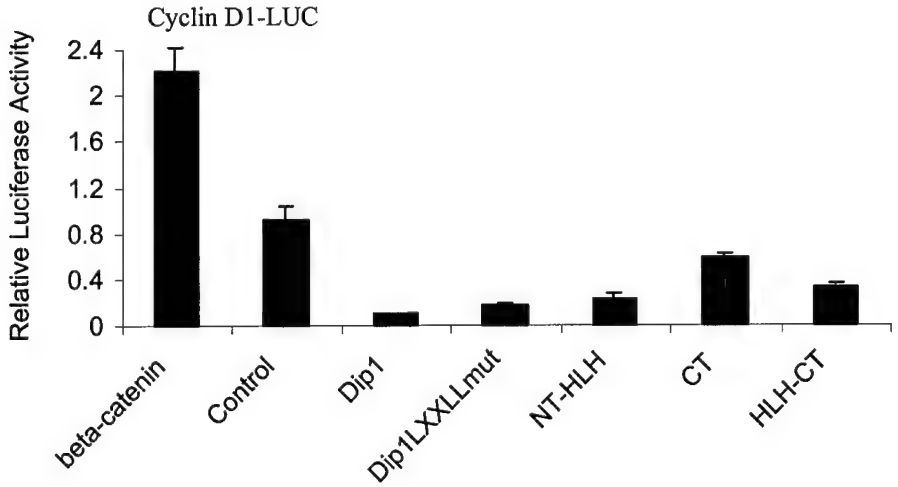
T1
T2
T3
T4
T5
T6
T7

B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Relative Luciferase Activity
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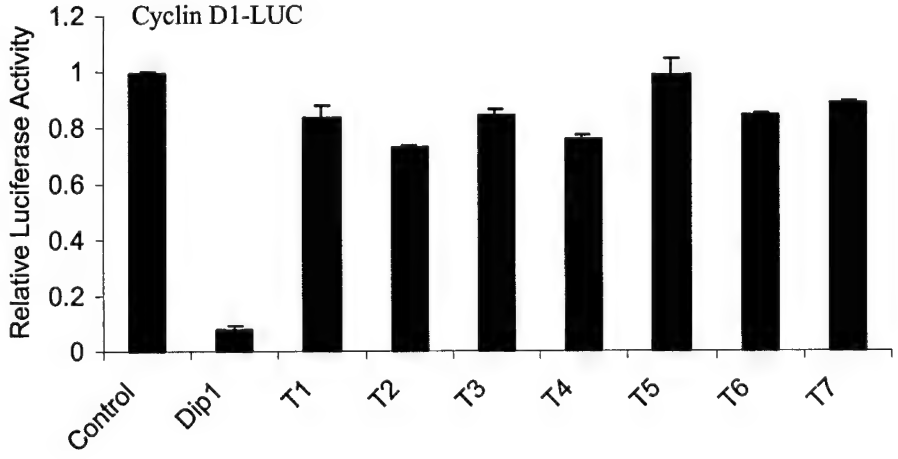
C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Relative Luciferase Activity
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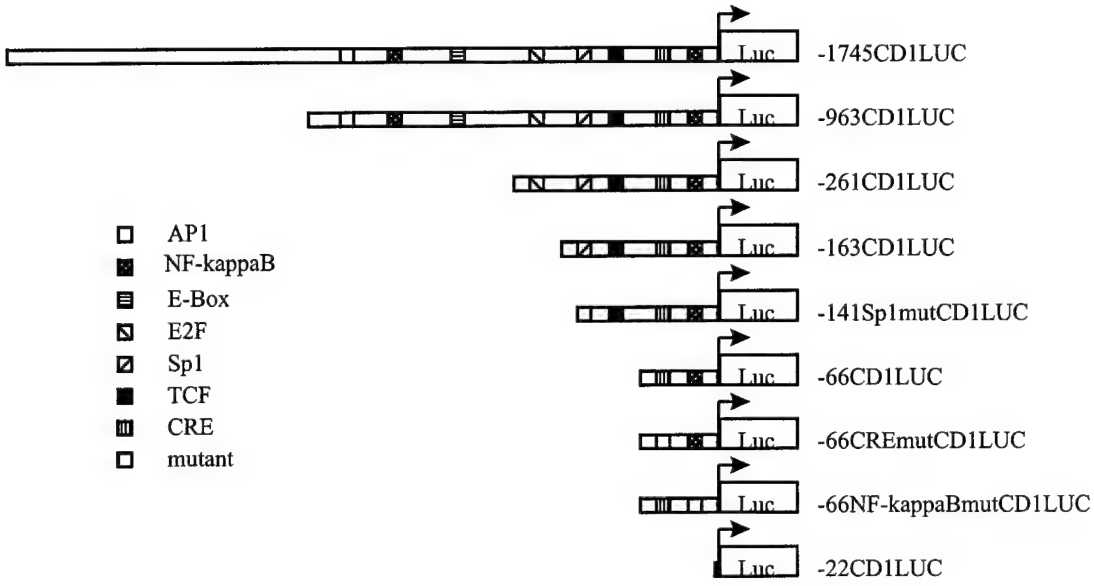
T7

Figure 5

A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TCF
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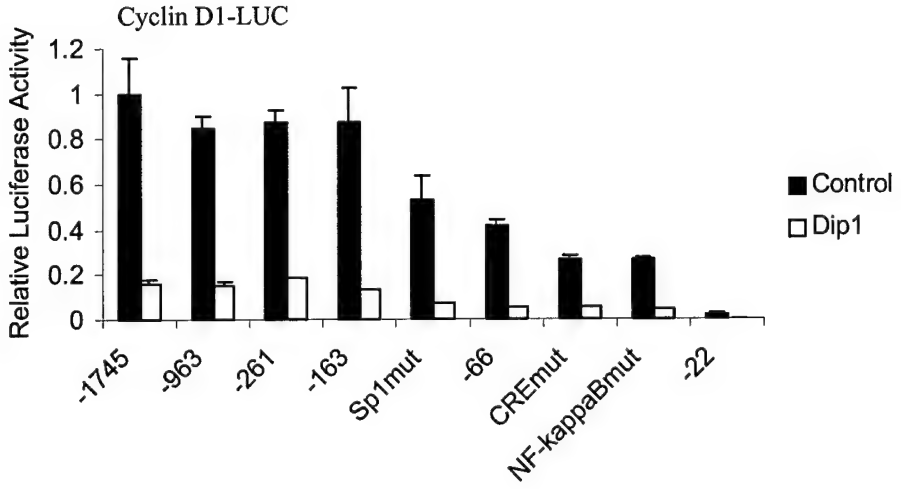
B


Cyclin D1-LUC
Relative Luciferase Activity
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1
0.8
0.6
0.4
0.2
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-261
-163
Sp1mut
-66
CREmut
NF-kappaBmut
-22
Control
Dip1

Figure 6

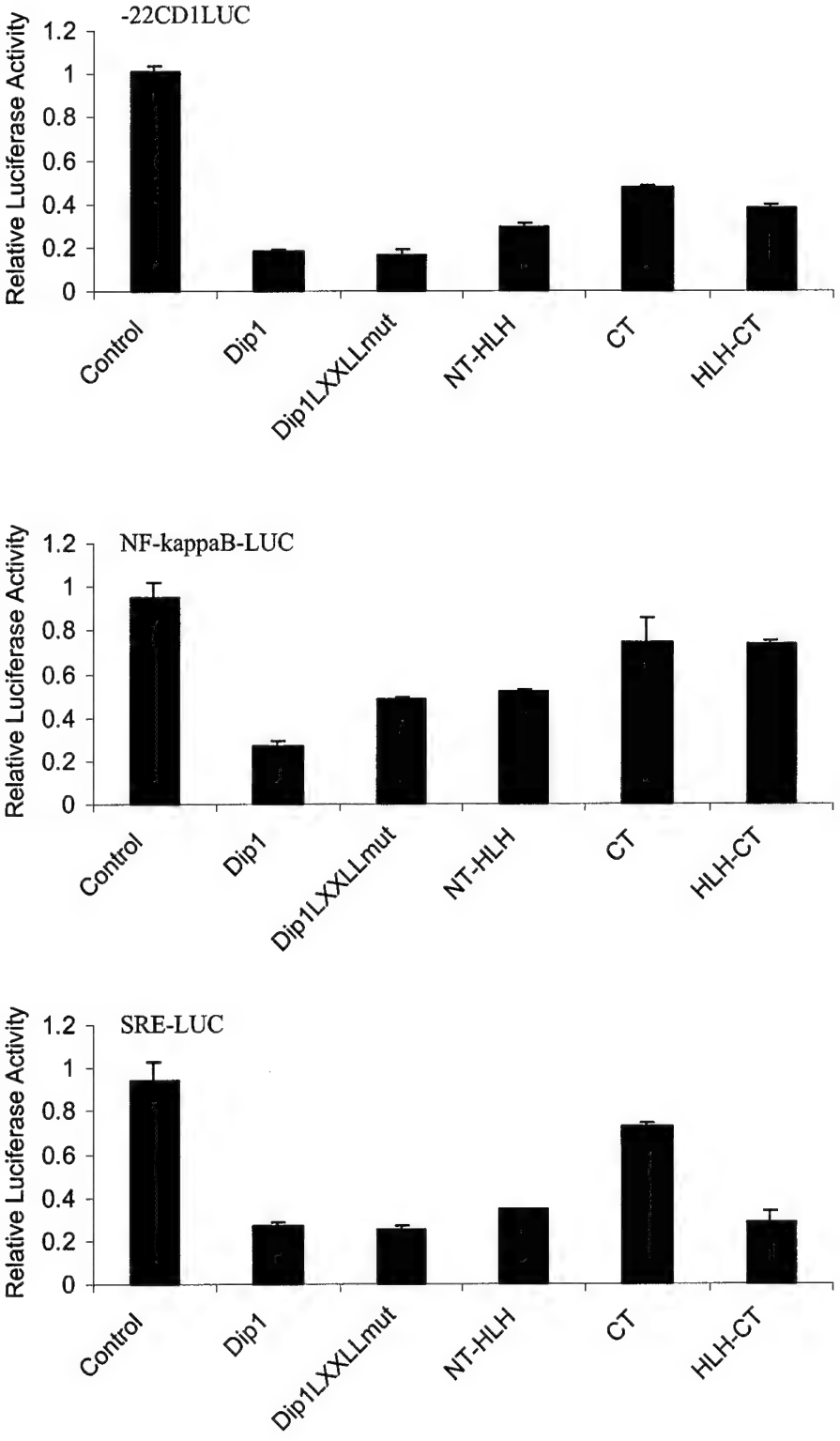
-22CD1LUC
Relative Luciferase Activity
1.2
1
0.8
0.6
0.4
0.2
0
Control
Dip1
Dip1LXXLLmut
NT-HLH
CT
HLH-CT
NF-kappaB-LUC
Relative Luciferase Activity
1.2
1
0.8
0.6
0.4
0.2
0
Control
Dip1
Dip1LXXLLmut
NT-HLH
CT
HLH-CT
SRE-LUC
Relative Luciferase Activity
1.2
1
0.8
0.6
0.4
0.2
0
Control
Dip1
Dip1LXXLLmut
NT-HLH
CT
HLH-CT

Figure 7

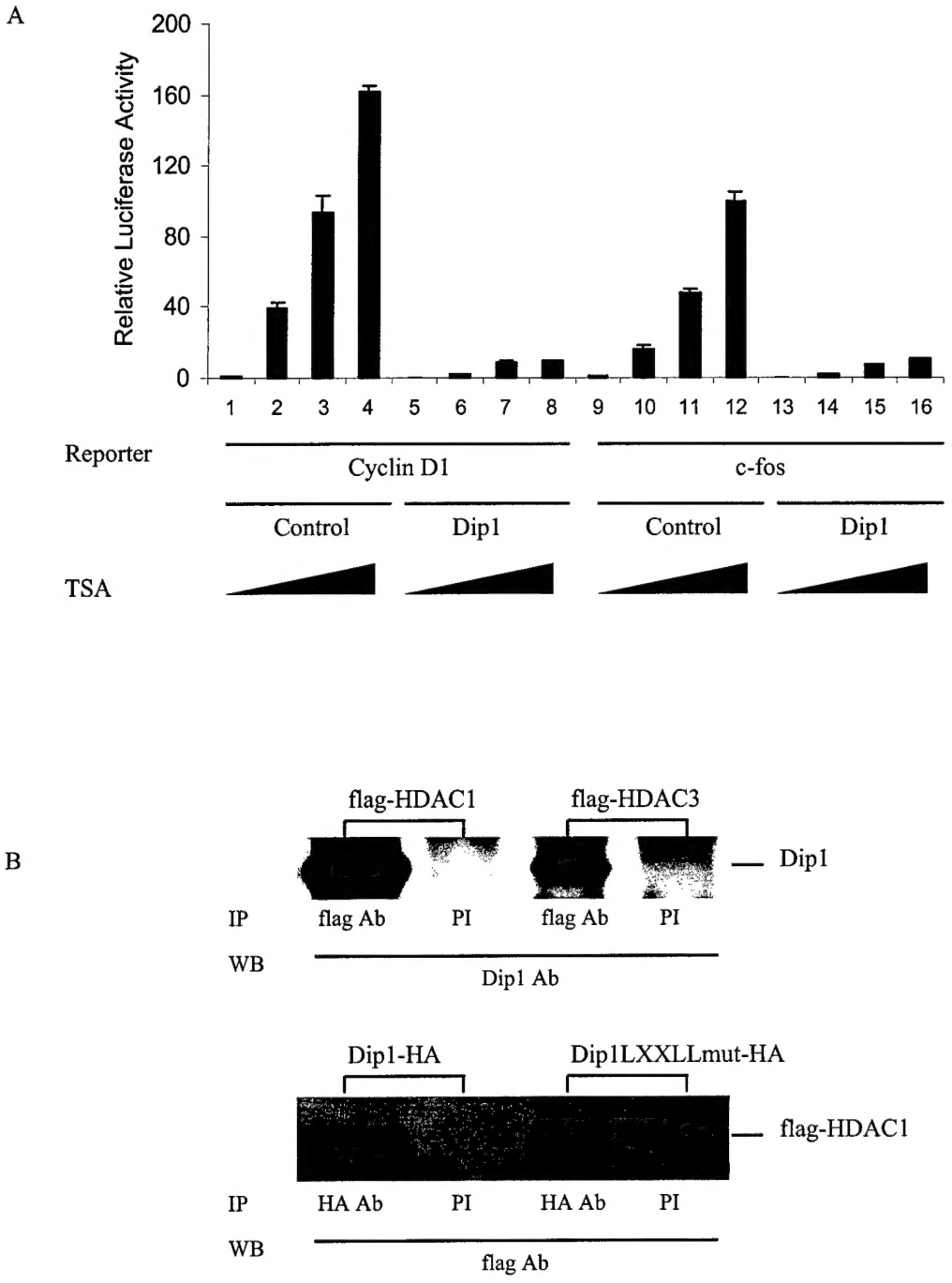
A
Relative Luciferase Activity
200
160
120
80
40
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Reporter
Cyclin D1
c-fos
Control
Dip1
Control
Dip1
TSA
B
flag-HDAC1
flag-HDAC3
Dip1
IP
flag Ab
PI
flag Ab
PI
WB
Dip1 Ab
Dip1-HA
Dip1LXXLLmut-HA
flag-HDAC1
IP
HA Ab
PI
HA Ab
PI
WB
flag Ab

Figure 8

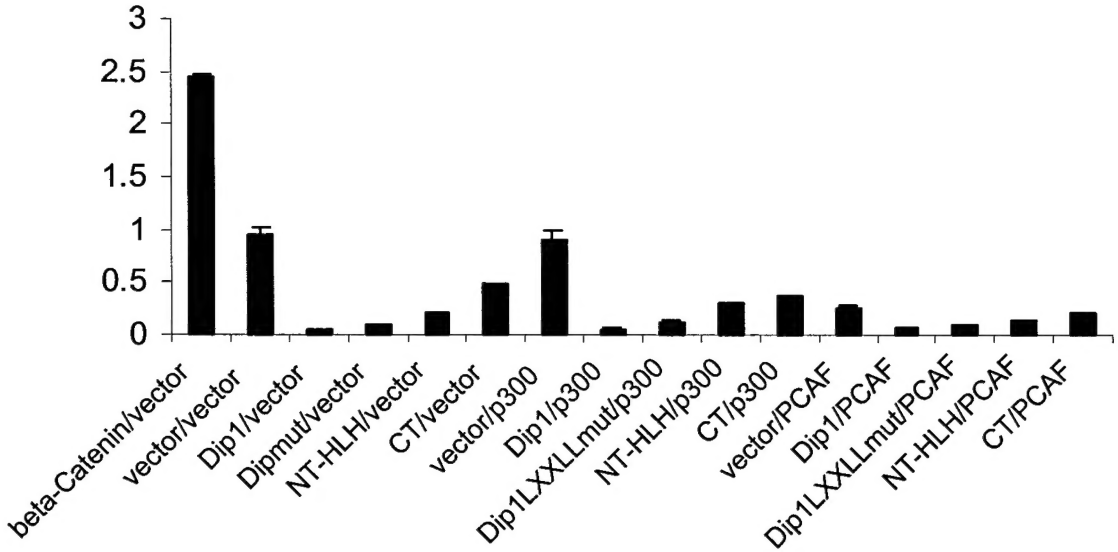
3
2.5
2
1.5
1
0.5
0
beta-Catenin/vector
vector/vector
Dip1/vector
Dipmut/vector
NT-HLH/vector
CT/vector
vector/p300
Dip1/p300
Dip1LXXLLmut/p300
NT-HLH/p300
CT/p300
vector/PCAF
Dip1/PCAF
Dip1LXXLLmut/PCAF
NT-HLH/PCAF
CT/PCAF

Figure 9

Figure 1. The nucleotide sequence of Dip1 cDNA and its deduced amino acid sequence. The cDNA contains a coding region of 1080bp, encoding a protein of 360 amino acids. The protein contains a putative leucine zipper (boldface and underlined), a HLH motif (underlined), a LXXLL motif (double underlined), and an acidic region (open box). A mutant was constructed in which the leucine residue at position 28 in the LXXLL motif was replaced by alanine. This mutant is designated "Dip1LXXLLmut".

Figure 2. Increased expression of Dip1 inhibits the growth of MCF7 cells. (A) MCF7 cells were plated ($1x10^5$ cell/10cm dish) and then transfected with either the empty vector or the vector containing Dip1 or Dip1LXXLLmut cDNA. The cells were then grown in selection medium containing hygromycin for two weeks and then fixed with 37% formaldehyde and stained with 5% Giemsa. (B) MCF7 cells stably transfected with the empty vector alone, Dip1 or Dip1LXXLLmut cDNAs were plated individually ($1x10^3$ cells/well in 96-well plates) and assayed for growth using the MTT assay every 24 hours.

Figure 3. Cell cycle analysis of exponentially growing cultures of vector control (A) and Dip1 overexpressing (B) MCF7 cells.

Figure 4. Effects of Dip1 on promoter-luciferase reporter assays in MCF7 cells. MCF7 cells were plated and then transiently transfected with vector, Dip1 or Dip1LXXLLmut plasmid DNA, together with Cyclin D1 or c-fos reporters. Cells extracts were assayed for luciferase activity 24 hours after transfection. Assays were repeated at least 3 times and gave similar results.

Figure 5. Dip1 truncation mutants and their effects on Cyclin D1 promoter activity in MCF7 cells. (A) A series of truncation mutants of Dip1 were created based on the domains in Dip1. (B) and (C) The effects of each truncation mutant on Cyclin D1 reporter activity were determined.

Figure 6. Effects of Dip1 on the transcription of a series of truncated and mutant forms of the Cyclin D1 promoter, shown in (A), when tested in transient transfection assays (B).

Figure 7. Effects of Dip1 and a series of derivatives of Dip1 (described in Fig. 5A) on the activities of the -22CD1LUC (A), NF-kappaB-LUC (B), and SRE-LUC (C) reporters in transient transfection assays in MCF7 cells.

Figure 8. Effects of Dip1 on histone deacetylase (HDAC). (A) MCF7 cells were transiently transfected with the indicated constructs. The cells were then incubated with increasing concentrations (50-200nM) of TSA, and extracts were prepared and assayed for luciferase activity after 24 hours. (B) Upper: MCF7 cells were transiently transfected with Dip1, flag-tagged HDAC1 or flag-tagged HDAC3 constructs as indicated. Total protein extracts were prepared 24 hours later and immunoprecipitated with flag antibody, and then western blotted by Dip1 antibody. Lower: Cells were transiently transfected with HA-tagged Dip1, HA-tagged Dip1LXXLLmut and flag-tagged HDAC1. Protein

extracts were immunoprecipitated with HA antibody and western blotted with flag antibody.

Figure 9. Neither p300 or PCAF reverse the inhibitory effects of Dip1 on Cyclin D1 reporter activity. MCF7 cells were transiently transfected with the indicated constructs and luciferase activities were assayed 24 hours after transfection.